Long-term treatment of polysaccharides-based hydrogel microparticles as oral insulin delivery in streptozotocin-induced type 2 diabetic mice

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ABSTRACT

To develop a more effective and safer drug for the treatment of type 2 diabetes mellitus (T2DM), polysaccharides- based hydrogel microparticles as oral insulin delivery was prepared and explored. This study was aimed to evaluate the antidiabetic effects and hypoglycemic mechanism with long-term administration(four weeks) of oral insulin hydrogel microparticles in type 2 diabetic mice on a model of diabetes using a high fat diet combined with streptozotocin. The results revealed that the long-term treatment of oral insulin polysaccharides-based hydrogel microparticles could significantly alleviate the symptoms of polyphagia, polydipsia, polyuria and weight loss in diabetic mice. Also, oral administration of insulin hydrogel microparticles could significantly reduce fasting blood glucose levels, ameliorate insulin resistance and increase insulin sensitivity in the mice with T2DM. The concentration of plasma TG, TC, LDL-C, FFA, BUN, CRE significantly decreased and the levels of HDL-C increased showed that insulin polysaccharides-based hydrogel microparticles were effective in regulating lipid metabolism and prevent diabetic nephropathy complication in diabetic mice. In addition, the supplementation of insulin hydrogel microparticles could significant improve the antioxidant capacity by increasing the level of SOD, CAT and decreasing the level of MDA, GPT, NO, TNF-α, and reverse histological deterioration of kidney and pancreas in diabetic mice. The above outcome concluded that insulin polysaccharides-based hydrogel microparticles may exhibit promising anti-diabetic activity and the potential to be a drug candidate for T2DM.

1. Introduction

Chitosan (CS) is a cationic, non-toxic, natural polysaccharide with high biocompatibility, biodegradability and pH-sensitivity, which has been generally used as carrier for the drug delivery of anticarcinogen, peptides, gene and protein [1–3]. In addition, CS can be easily converted to hydrogels, thus it is urgently necessary exploiting new-type chitosan based hydrogel, not only has the excellent original properties of hydrogel, but also has the great ability for maintaining sustainable effects of these drugs. β-cyclodextrin (β-CD) could accommodate a variety of lipophilic drugs due to its hydrophilic outer surface and lipophilic
inner cavity, which could be used as drug formulations with significantly improved bio-availability of active compound along with reduction of different adverse effects [4–6]. Chitosan hydrogels have suitable space structure, and introducing β-CD inclusion compound into chitosan hydrogels may be an available way to improve the properties of chitosan hydrogels. Recently, efforts have been made to synthesize CS/CD hybrid materials, which simultaneously offer the excellent mucoadhesive properties of CS and the high inclusion of CD [7–11]. According to the advantages of both β-CD and CS, combining β-CD with CS as a drug carrier which CS can protects drug from the harsh environment of the gastrointestinal environment, safeguard the encapsulated drug on the
β-CD from the pH environment and enzyme degradation. As the interests in design hydrophilic/hydrophobic drug and macromolecular drug for oral delivery have been increased, and drug delivery systems based on CS and CD NPs have been widely investigated.

Diabetes mellitus (DM) is a worldwide metabolic/endocrine disease [12–14]. DM may be categorized into type 1 and type 2 diabetes, type 1 diabetes mellitus (T1DM, known as insulin dependent DM) and type 2 diabetes mellitus (T2DM, known as non-insulin dependent DM). Around 90–95 % of diabetic individuals are diagnosed to be T2DM, which is characterized by abnormal metabolism of biomolecules in the body, due to chronic hyperglycemia and insulin resistance caused by reduced sensitivity of insulin resistance in insulin-sensitive tissues, which will be at risk of developing diabetes-related complications [15–18]. The frequency of T2DM will escalate worldwide, due to the continuously increased number of elderly people and unhealthy diet, lacking of physical activity, and smoking. Therefore, the development of better treatments and effective anti-diabetic agents for T2DM has become imminent. Although clinical

Abbreviations: CMC, carboxymethyl chitosan; CMCD, carboxymethyl β-cyclodextrin; NHS, N-hydroxysuccinimide; EDC, 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimidehydrochloride; INS, insulin; STZ, streptozotocin; DM, diabetes mellitus; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; FBG, fasting blood glucose; OGTT, oral glucose tolerance test; AUC, area under the curve; FINS, fasting serum insulin; HOMA-IR, homeostasis model assessment of insulin resistance; ISI, insulin sensitivity index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SOD, superoxide dismutase; MDA, malondialdehvde; CAT, catalase; GPT, glutamic-pyruvic transaminase; BUN, blood urea nitrogen; CRE, creatinine; FFA, free fatty acid; NO, nitric oxide; HE, hematoxylin-eosin; SD, standard deviations.

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pharmacological treatment of diabetes is predominantly based on oral hypoglycemic agent, the sustaining use of chemical synthetic anti-diabetic drugs lead to cytotoxicity and associated side effects, including hypoglycemia, fluid retention, osteoporosis and heart failure, which limit their clinical applications [19].

To date, insulin is commonly administered by subcutaneous injection for the treatment of DM. However, daily injections of insulin have many disadvantages, such as hypoglycemic events, unwanted pain, allergic reactions, discomfort, peripheral hyperinsulinemia, and other side effects [20–24]. Among alternative insulin administration routes, the oral route seems to be the more advantageous for long-term administration. Thus, numerous tries have been made to open up effective oral delivery systems for insulin. Notably, they exhibit many favorable properties, including better safety, biocompatibility, storage stability, and physiological stability [21,25–28]. Nevertheless, as far as we know, oral insulin drug delivery has not been inquired into in any depth regarding its effects on hyperglycaemic and hyperlipidemic in streptozotocin (STZ)-induced type 2 diabetic mice. In our previous study, carboxymethyl β-cyclodextrin (CMCD) grafted carboxymethyl chitosan (CMC) hydrogel-based microparticles as an oral insulin delivery system were prepared and characterized [29]. This paper aimed to analyse the effect of hypoglycemic and multiple biological activities with long-term (four weeks) administration of oral insulin hydrogel microparticles in type 2 diabetic mice, which can help for future pharmacological studies of T2DM.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

CMCD (99.3 %, purity) was purchased from Shandong Binzhou Zhiyuan Biotechnology Co., Ltd., China. CMC (carboxylation degrees ≥80 %) was obtained from Yuanye Biotechnology Co., Ltd., Shanghai, China. *N*-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) were supplied by Shanghai Macklin Biochemical Co., Ltd., China. Insulin (100IU/mL), total cholesterol (TC, Lot.No.20191130), high-density lipoprotein cholesterol (HDL-C, Lot.No.20191113), low-density lipoprotein cholesterol (LDL-C, Lot.No.20191117), malondialdehvde (MDA, Lot.No.20191206), triglyceride (TG, Lot.No.20191128), superoxide dismutase (SOD, Lot. No.20191114), catalase(CAT, Lot.No.20191117), creatinine(CRE, Lot. No.20191024), glutamic-pyruvic transaminase(GPT, Lot. No.20191106), blood urea nitrogen(BUN, Lot.No.20191210), free fatty acid (FFA, Lot.No.20191111), nitric oxide (NO, Lot.No.20191103) kits and insulin ELISA(Lot.No.20190521), TNF-α ELISA kits (Lot. No.20190907) were provided from Beijing Solarbio Science & Technology Co., Ltd., China. All other reagents used were commercially available and were of analytical grade. The above materials were used from the indicated sources without any purification.

2.1.2. Experimental animals

Male Kunming mice (2~3 weeks, 16− 18 g) were obtained from Hunan SJA Laboratary Animal Co., Ltd., China. Mice were housed in the Animal Experimental Center (University of Shantou) controlled at a relative humidity of 55 ± 10 % and constant temperature of 22 ± 1 °C with a 12 h light-dark cycle. Blood glucose meter was purchased from Sinocare Inc. (Hunan, China). Streptozocin (STZ) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).The high-fatdiet was purchased from Beijing BoAiGang Technology Co. Ltd., China. *2.2. Preparation of Insulin/CMCD-g-CMC hydrogel microparticles*

CMCD was grafted to CMC by EDC/NHS-mediated amidation reaction. CMC (4.0 g, 17 mmol) and CMCD (4.0 g, 3.3 mmol) were dissolved completely in distilled water (100 mL) containing EDC (3.68 g, 19.2 mmol) and NHS (2.21 g, 19.2 mmol). The viscous solution was precipitated in methanol, after under gentle stirring at 25 °C for 24 h. Soak the product in distilled water to make it expand, and the obtained hydrogel was dialyzed against distilled water for 3 days utilizing a dialysis membrane (Molecular cutoff: 2 kDa) to remove unreacted reagents. The dialyzed hydrogel was precipitated by methanol and then dried at 40 °C under reduced pressure. Finally, the resultant solid particles were cut and screened through a 20-mesh filter using a PLC-2 M plastic cutting mill (Osaka Chemical Co., Japan) to obtain white granular CMCD-g-CMC hydrogel microparticles. For the preparation of drug- loaded hydrogels, insulin was loaded into the hydrogels by a swelling-diffusion method. Further, the dried and weighed hydrogel microparticles were immersed into different concentrations of insulin solution and allowed to swell. This step was performed in a metal bath oscillator (100 rpm) at 37 °C for 24 h. After swelling equilibrium, the insulin/ CMCD-g-CMC hydrogel microparticles (INS/CMCD-g-CMCs)were taken out and collected for future use.

2.3. Induction of experimental type 2 diabetic mice and administration of drug

There was an adaptation period for three days, and after that, there mice were divided into two groups as follows; 60 in the STZ-induced diabetic group and 10 in the uninduced group. All the STZ-induced diabetic mice were fed with a high-fat diet (65 % normal chow diet, 10 % lard oil, 20 % sucrose, 2.5 % cholesterol, 1% carbohydrate, 1% sodium deoxycholate and 0.5 % cellulose)to the end of the experiment.

Table 1
Animal grouping and treatments.

Groups	Diets	STZ induction	Treatment	Dosage/ bw*
Control	Normal diets	_	Saline	0.1

Model	High-fat diet	Three times	Saline
PC	High-fat diet	Three times	S.c.Insulin
NC	High-fat diet	Three times	Oral insulin
Gel-L	High-fat diet	Three times	INS/CMCD-g-CMCs
Gel-M	High-fat diet	Three times	INS/CMCD-g-CMCs
Gel-H	High-fat diet	Three times	INS/CMCD-g-CMCs

* bw denotes the body weight of mice.

To induce a type 2 diabetic mice model, the STZ-induced mice were fed with a high-fat diet for 4 consecutive weeks, and then injected intraperitoneally three times with a freshly prepared STZ (60 mg/kg; using a small number of multiple injections) after 12 h fasting. The STZ solution was diluted with freshly prepared citric acid buffer (0.1 M) to maintain a pH of 4.5, dilute it when it will be used [30,31]. After injection, mice were further fed with a high-fat diet for another 4 weeks. Blood glucose concentration was determined with a handheld glucometer. The mice were fasted overnight (8 h) to the experiment, but were allowed free access to water. Fasting blood glucose (FBG) level of mice was tested by taking a drop of blood from the via tail vein by a handheld glucometer every week. Two FBG concentrations ≥16.7 mol/L were helpful to establish the stability of the mouse model of type 2 diabetes, so, it was selected for further pharmacological research.

After establishment of the diabetic mice model, the 10 uninduced mice as the normal control group (control group) and the diabetic mice were then divided into 6 groups, with 10 mice in each group (Table 1). Model group animals received intragastric administration of 0.9 % normal saline as diabetic control and control group animals were treated with the same volume of normal saline. PC group animals received 5 IU/ kg free insulin solution as a positive control (subcutaneous injection, S.c. Insulin), whereas NC group animals received 100 IU/kg free insulin solution orally as insulin control. Three groups (Gel-L, Gel-M, and Gel- H) of diabetic mice received INS/CMCD-g-CMCs solution for intragastric administration at a dose of 50, 75 and 100 IU/kg, respectively. All the mice underwent the treatment for four consecutive weeks by gavage/injection once a day. All experiments were approved by the institutional animal care and use committee.

2.4. Measurement of body weight, food intake and water intake, FBG

The mice were fed and watered freely under different cage conditions, and were given a daily dose of medicine for 4 weeks. The weight of each mouse was measured weekly. During the test, the food and water in each fresh water container was broken down and recorded (the weight difference before and after the test was calculated). The total food intake and water consumption of each cage were monitored regularly and the average values for each mouse calculated.

Considering the effect of anaesthetics on blood glucose levels, no anaesthesia was used during animal experiments. Eight hours after fasting, the FBGs of the mouse in each group were determined weekly until mice were sacrificed.

2.5. Oral glucose tolerance test (OGTT)

On the last week of the experimental period, OGTT was performed using a glucose aqueous solution (2 g/kg bw) orally administrated to all mice that were fasted overnight. The tail vein blood was subsequently collected at 0, 30, 60 and 120 min. The blood glucose was monitored using the glucometers to calculate the OGTT values. The trapezoidal rule was used to calculate the curve under the area (AUC) using the following Eq. 1[32]:

$$AUC\ (mmol\ minL) = (a+2b+3c+4d)/4 \quad (1)$$

Note: a, b, c and d are the blood glucose values after 0, 30, 60, 120 min, respectively, after being fed glucose.

2.6. Effects of INS/CMCD-g-CMCs on organ index in diabetic mice

At the end of experimental period, food was withdrawal from the mice overnight. Then, the liver, kidney, pancreas and spleen was removed immediately after the mouse sacrificed of cervical dislocation, washed with cold physiological saline, then absorbed excess water by filter paper, finally, collected and weighted. One part of liver and kidney were preserved in 10 % buffered formalin and stored at − 80 °C for tissue lipid estimations. One part of kidney and pancreas were reserved for

subsequently histopathological evaluation. The organ index was the ratio of wet weight of the organ tissue to body weight as follows Eq. 2 [30]:

Organ index (%)=$Weight_{organ}$/$Weight_{mouse}$×100 % (2) Note: $Weight_{organ}$ is the organ weight after slaughter, $Weight_{mouse}$ is the last weight of a mouse before slaughter.

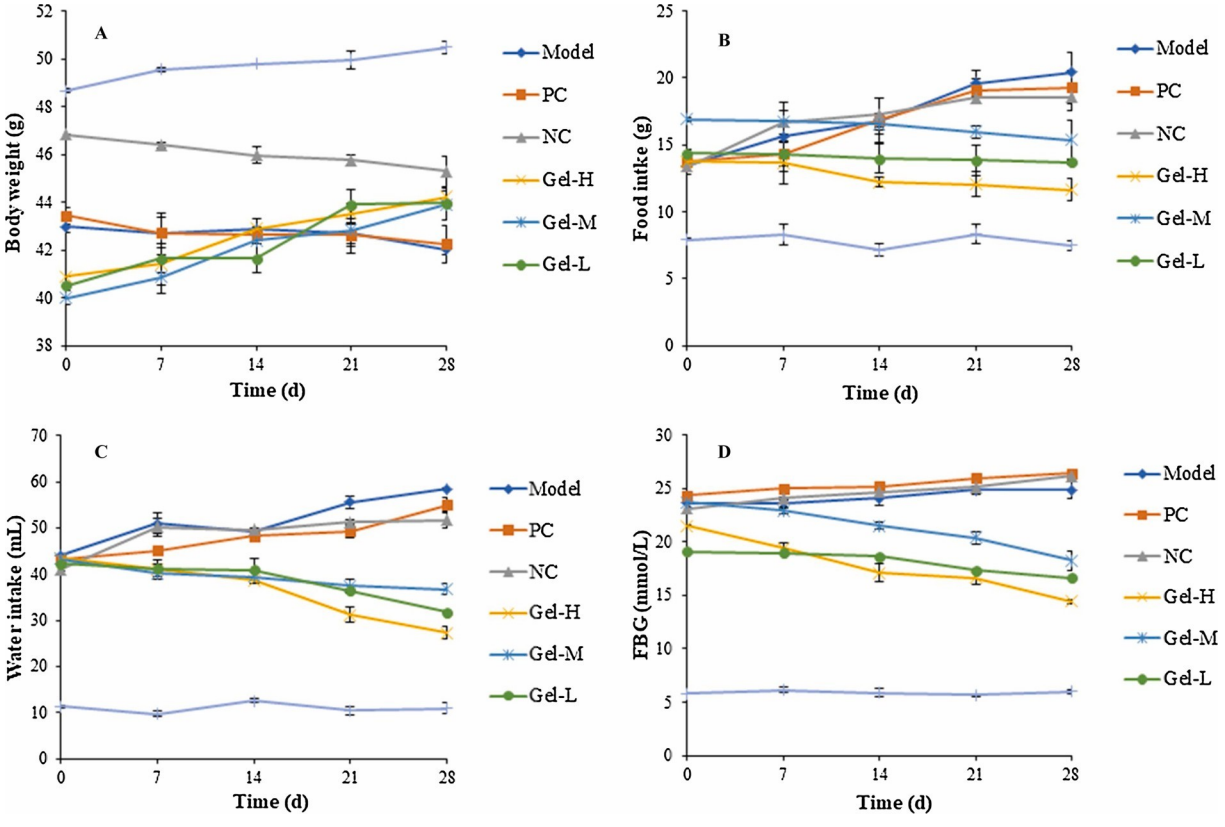

Fig. 1. Effects of INS/CMCD-g-CMCs on(A) body weight; (B) mice mean food intake; (C) mice mean water intake; (D) FBG in diabetic mice.

2.7. Biochemical analysis

Blood samples were collected by eyeball bleeding of the animals into serum separator tubes and centrifuged (4 °C, 3000 rpm, 10 min) to obtain serum. The serum was transferred into fresh tubes and stored at − 80 °C until use for further biochemical analysis.

Fasting serum insulin (FINS) was determined in strict accordance with the instructions of the insulin ELISA kit by using enzyme linked immunosorbent assay. Homeostasis model assessment of insulin resistance (HOMA-IR) is an indicator to assess the insulin resistance level as shown in Eq. 3 [33]. And insulin sensitivity index (ISI) were calculated according to the following Eq. 4[34].

$$\text{HOMA-IR}=(\text{FBG} \times \text{FINS})/22.5$$

$$\text{ISI} = \text{Ln}(\text{FBG} \times \text{FINS})-1 \quad (4)$$

Note: FBG is last fasting blood glucose of a mouse before slaughter (mmol/L), and FINS is the value of fasting serum insulin (mIU/L). Commercial kits were used to determine the levels of TC, TG, HDL-C, LDL-C, BUN, CRE and FFA in the serum. One part of liver was homogenized and centrifuged (3000×g, 10 min, 4 °C) and collected the supernatant for determination. MDA, SOD, CAT, GPT and NO activities were measured by corresponding specific assay kits. The operation and analysis were according to the manufacturer's instructions. Meanwhile, one part of kidney was homogenized and centrifuged (3000×g, 10 min, 4 °C) and collected the supernatant for estimations. TNF-α level was determined in strict accordance with the instructions of the TNF-α ELISA kit by using enzyme linked immunosorbent assay. All results were obtained using a multi-wavelength microplate reader (ThermoElectron Corp., Boston, MA, USA).

2.8. Histopathological studies

The kidney and pancreas sections were fixed with 10 % formaldehyde dewaxed and dehydrated with ethanol (50–100 %, 10 % increments) and paraffin-embedded, sectioned at 4− 5 μm by rotary microtome, then dewaxed and rehydrated [35–37].

Sections were then stained with hematoxylin-eosin (H&E) and fixed on slides for morphological observation through an optical microscope (OLYMPUS CH20, Japan) and were photographed (400×). The histologic scores were evaluated as described previously [38,39].

2.9. Statistical analysis

All the experimental data were expressed as means ± standard deviation (SD) for ten mice in each group. Statistically significant differences among groups were analyzed using one-way ANOVA (SPSS software, version 20.0, China) with LSD post-hoc test, and values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Effect of INS/CMCD-g-CMCs on the growth performance in diabetic mice

The behavioral observations of mice were obvious difference, the normal control mice exhibited a good mental condition, activity, smooth fur, and steady weight gain. In stark contrast, while after treatment (high-fat diet and low-dose STZ injection combined induction), the model diabetic mice were observed to have dark yellow fur and poor mental state, with increased food and water intake, increased urination, and decreased body weight.

As shown in Fig. 1 A, compared with normal mice, mice injected with STZ had significantly lower body weight at the beginning, it was normal experimental phenomenon because STZ injections can cause weight loss at first. After 4 weeks' experiment, the body weight of the control group

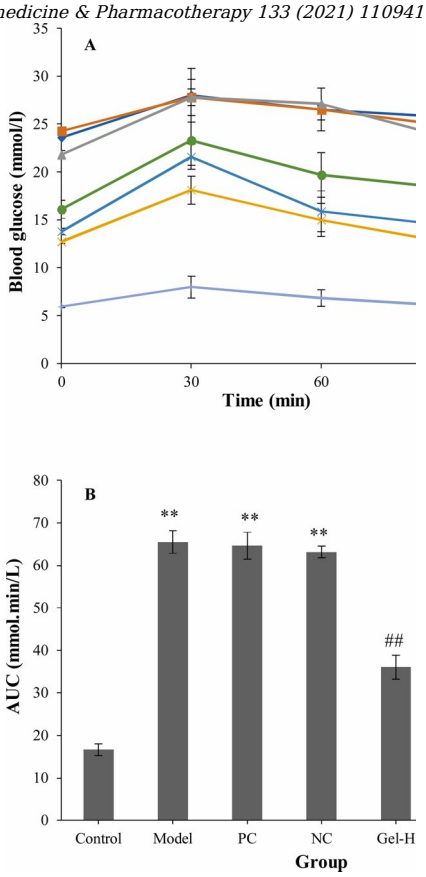

Fig. 2. (A) Effects of INS/CMCD-g-CMCs on glucose tolerance test; (B) Area under the curve in diabetic mice. Data are expressed as means ± SD (n = 10). *P < 0.05, **P < 0.01vs control; #P < 0.05, ##P < 0.01vs model.

continued to increase steadily. Compared with control group, the body weight of model, PC and NC groups kept dropping, which was a symptom of increasing diabetes. Gel-L, Gel-M and Gel-H groups showed no significant downward trend but exhibited obvious gain in bodyweight. This experiment had showed that the long-term treatment with different doses of INS/CMCD-g-CMCs were effective on alleviating the loss of body weight.

3.2. Effect of INS/CMCD-g-CMCs on diets in diabetic mice

Model diabetic mice usually have one clear sign of the disease, which is crapulent. Fig. 1B showed that the food intake of the control group was constant with occasional fluctuations. The food intake of model, PC and NC groups showed the tendency of increasing gradually. However, Gel-L, Gel-M and Gel-H groups had a significant reduction in food intake. Compared with the Gel-administered groups, the food intake of the model, PC and NC groups were higher. As shown in Fig. 1C, the mice in the control group drank normal water, with an average of 10.9 ± 0.30 mL/d/mouse. However, the water consumption of diabetic mice increased sharply, and the average water consumption of the diabetic mice was 43.5 ± 1.34 mL/d/mouse, which was more than 4 times greater than the BC group. The water consumption of the model, PC and NC groups continued to increase gradually. Compared with these groups, Gel-L, Gel-M and Gel-H groups showed the tendency of decreasing slightly first and then a significant downward trend. And the polydipsia symptoms of the Gel-H group had the greatest relief. Taken together, these data suggest that oral administration of INS/CMCD-g- CMCs for 28 days continuously, the symptoms of overeating and drinking are effectively alleviated, and the effect on the Gel-H group was the greatest, which showed a significant reduction the intake of water

Table 2
Effect of INS/CMCD-g-CMCs on organ index, fasting serum insulin level, lipid metabolism and oxidative stress in diabetic mice.

Parameter	Control	Model	PC	NC	Gel-H	Gel-M	Gel-L
Organ index(%)							
Liver	4.51 ± 0.21	5.98 ± 0.64**	5.98 ± 0.45**	5.98 ± 0.45**	5.33 ± 0.22**##	5.54 ± 0.47**#	5.56 ± 0.66**#
Kidney	1.45 ± 0.10	1.75 ± 0.10**	1.73 ± 0.18**	1.66 ± 0.03**	1.51 ± 0.04##	1.58 ± 0.08##	1.58 ± 0.14##
Pancreas	0.68 ± 0.07	0.52 ± 0.10**	0.52 ± 0.02**	0.53 ± 0.03**	0.59 ± 0.09*#	0.61 ± 0.06*#	0.61 ± 0.06*#
Spleen	0.33 ± 0.08	0.19 ± 0.01**	0.22 ± 0.01**	0.21 ± 0.04*	0.31 ± 0.06##	0.29 ± 0.07#	0.27 ± 0.04#
Fasting serumvel							
insulin le	0.29 ± 0.02						
FINS (ng/mL)	1.61 ± 0.54	3.14 ± 0.52**	2.98 ± 0.31**	2.93 ± 0.91**	1.80 ± 0.44**##	1.86 ± 0.82**##	1.88 ± 0.22**##
HOMA-IR	− 2.26 ± 0.52	73.58 ± 7.91**	74.18 ± 6.18**	72.31 ± 3.38**	24.46 ± 2.35**##	32.06 ± 2.46**##	29.99 ± 3.39**##
ISI		− 7.51 ± 0.81**	− 7.58 ± 0.81**	− 7.55 ± 0.62**	− 5.87 ± 0.75**##	− 6.37 ± 0.85**##	− 6.21 ± 0.25**##
Lipid metabolism							
TC (umol/L)	9.71 ± 0.01	26.61 ± 0.04**	22.24 ± 0.06**##	23.08 ± 0.05**##	12.31 ± 0.03**##	18.90 ± 0.05**##	18.91 ± 0.05**##
TG (mg/L)	1.10 ± 0.01	11.00 ± 0.02**	12.33 ± 0.01**##	11.20 ± 0.01**##	7.35 ± 0.03**##	8.50 ± 0.02**##	9.60 ± 0.01**##
HDL-C (mmol/L)	4.88 ± 0.42	2.72 ± 0.48**	2.47 ± 0.39**##	2.90 ± 0.26**	3.78 ± 0.67**##	3.67 ± 0.42**##	3.53 ± 0.48**##
LDL-C (mmol/L)	0.34 ± 0.17	3.08 ± 0.16**	2.84 ± 0.24**##	2.96 ± 0.07**	0.76 ± 0.16**##	0.84 ± 0.24**##	1.08 ± 0.59**##
FFA (mmol/L)	0.15 ± 0.01	1.60 ± 0.17**	1.53 ± 0.20**	1.41 ± 0.11**##	0.16 ± 0.24##	0.20 ± 0.15##	0.27 ± 0.15**##
Oxidative stress							
MDA (nmol/g)	8.37 ± 0.01	25.87 ± 0.01**	26.39 ± 0.01**##	24.36 ± 0.01**##	12.45 ± 0.05**##	14.70 ± 0.01**##	19.51 ± 0.01**##
SOD (U/g)	278.17 ± 2.19	154.41 ± 4.51**	175.73 ± 2.32**##	197.07 ± 4.85**##	244.73 ± 8.24**##	227.28 ± 3.82**##	212.73 ± 4.98**##
CAT (U/g)	358.02 ± 3.19	257.04 ± 1.06**	279.99 ± 8.71**##	275.40 ± 2.74**##	333.43 ± 8.91**##	321.30 ± 1.82**##	316.71 ± 1.52**##
GPT (U/g)	10.90 ± 2.95	30.69 ± 0.52**	29.28 ± 6.72**	27.09 ± 7.13**##	18.25 ± 2.08**##	19.68 ± 0.32**##	20.84 ± 4.04**##

Data are expressed as means ± SD (n = 10). *P < 0.05, **P < 0.01vs control; #P < 0.05, ##P < 0.01vs mode.

and food.

3.3. Effect of INS/CMCD-g-CMCs on FBG in diabetic mice

Fig. 1D presented the FBG of mice in all groups. It can be seen that the mean value of FBG in the BC group is 5.83 ± 0.23 mmol/L, which was always normal and stable. However, compared with the normal control group, the FBG of the diabetic mice was significantly higher, which was mainly manifested as glucose metabolism disorder. After 4 weeks of treatment, the FBG of model, PC and NC groups showed no decline but the trend is rising within a narrow range. All of the Gel-L, Gel-M and Gel-H groups displayed significant anti-hyperglycemic effects on diabetic mice. After 7 day, a significant change was observed and the FBG showed a steady decrease from the 14th to 28th days. With an increase of INS/CMCD-g-CMCs concentration, the effect on inhibiting blood glucose elevation was more obvious.

3.4. Effect of INS/CMCD-g-CMCs on OGTT

OGTT is a generally used way for diagnosing early diabetes and metabolic diseases, which has been also used to estimates of the ability to regulate blood glucose [34,36]. As shown in Fig. 2A, after taking glucose orally, the blood glucose level of control group increased significantly, decreased gradually after 30 min, and returned to the basic level after 120 min. However, the glucose tolerance of the model, PC and NC groups was severely damaged, which were abnormal, and the blood glucose level of these groups were consistently maintained in a high level throughout the whole time. Compared to the model, PC and NC groups, the blood glucose of the Gel-L, Gel-M and Gel-H groups was always much lower.

Administration caused increase sharply in blood glucose level within 30 min with a decline to original level in a short time. The utilization of glucose oxidation in tissues was enhanced due to the decrease of gluconeogenesis, and this result in oxidative stress which showed as an increase of glucose tolerance.

The area under the curve (AUC) is indirectly proportional to the glucose tolerance. In Fig. 2B, the AUC of model, PC and NC groups were the highest, which suggested that the glucose tolerance was severely deteriorated. Furthermore, the AUC of all the Gel-treated groups showed statistically significant dose-dependent differences. Briefly, these results suggested that INS/CMCD-g-CMCs could effectively reduce the burden on the islets cells due to slow down the absorption rate of glucose as a result of improving the impaired glucose tolerance of diabetic mice and maintained glucose homeostasis.

3.5. Effect of INS/CMCD-g-CMCs on liver, kidney, pancreas, and spleen weights in diabetic mice

As shown in Table 2, compared with the control group, the liver and kidney indexes of each diseased group (model, PC and NC groups) were significantly higher, which was as a result of the swelling of liver and kidney in diabetic mice. After being fed INS/CMCD-g-CMCs, the liver and kidney indexes of each Gel group were lower than those of the model, PC and NC groups. Compared with the other groups, the Gel-H group was the closest to the control group. INS/CMCD-g-CMCs inhibited the growth of the liver and kidney index in diabetic mice and had a role in protecting the liver and kidney. Additionally, compared with the control group, statically decrease of pancreas and spleen indexes in the diabetic mice was observed. After four weeks of oral administration, the weights of pancreas and spleen were restored to normal level gradually. The changes in organ indexes indicated that INS/CMCD-g-CMCs could inhibit diabetes-induced organ damage obviously and effectively protect the organs in diabetic mice.

3.6. Effect of INS/CMCD-g-CMCs on FINS, HOMA-IR and ISI in diabetic mice

Table 2 showed the FINS, HOMA-IR and ISI of mice in all the groups. The FINS and HOMA-IR of the model, PC and NC groups were significantly ($P < 0.01$) higher than the control group. Treatment with INS/ CMCD-g-CMCs, the FINS and HOMA-IR levels of each Gel-treated group showed a significant ($P < 0.01$) reduction in the index, which indicated that INS/CMCD-g-CMCs have significant alleviation effects on insulin resistance. As the results of ISI, compared with that in control group, the insulin sensitivity was low in the mice with diabetes. Oral administration of INS/CMCD-g-CMCs for 4 weeks enhanced insulin sensitivity with a slightly dose-dependent manner. From the above, it could be concluded that INS/CMCD-g-CMCs was alleviated disrupted insulin resistance, glucose homeostasis and the amelioration of hyperglycemia situation in type 2 diabetic mice.

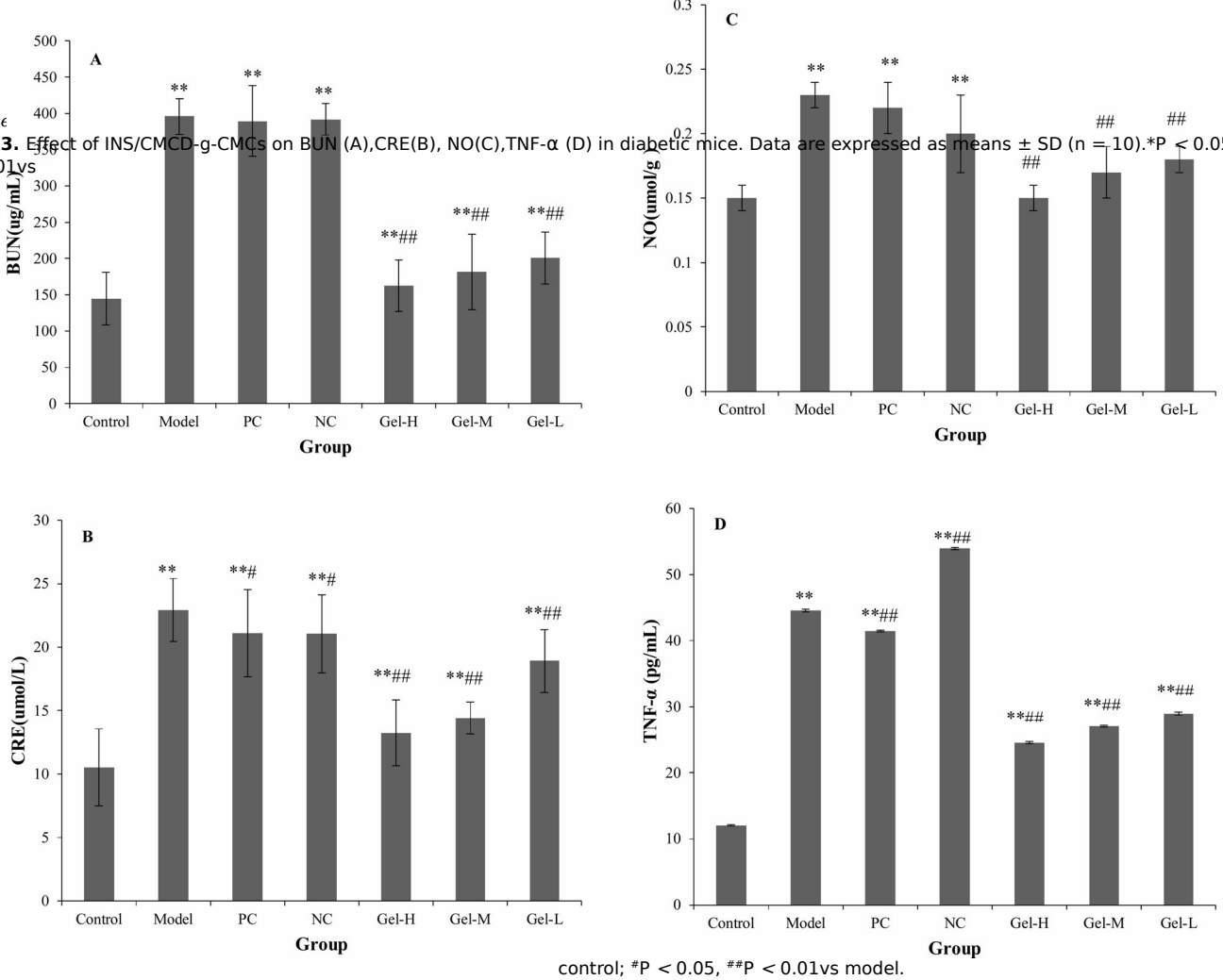

Fig. 3. Effect of INS/CMCD-g-CMCs on BUN (A),CRE(B), NO(C),TNF-α (D) in diabetic mice. Data are expressed as means ± SD (n = 10).*P < 0.05, **P < 0.01vs control; #P < 0.05, ##P < 0.01vs model.

3.7. Effect of INS/CMCD-g-CMCs on lipid metabolism and complications in diabetic mice

The lack of insulin causes the decomposition of a large number of fat tissues, resulting in lipid metabolism disorders [40,41]. As shown in Table 2, after STZ treatment, the level of plasma TG, TC, LDL-C, and FFA were increased significant ($P < 0.01$), but the levels of HDL-C decreased significantly ($P < 0.01$). As compared to control group, whereas diabetic mice treated with oral administration of INS/CMCD-g-CMCs for 4 weeks showed the significance ($P < 0.01$) reduction in the levels of TG, TC, LDL-C, FFA and increased level of HDL-C compared to model, PC and NC groups.

The high level of BUN and CRE is a risk essential for diabetic nephropathy, and it also is one of the diabetic mellitus chronic complications [19]. Fig. 3A showed the diabetic mice group's BUN level was substantially ($p < 0.01$) increased, compared with the control group. However, compared with that of model, PC and NC groups, the Gel-treated groups, especially Gel-H group were decreased in BUN content. Fig. 3 B revealed that significant ($p < 0.01$) decreased in CRE levels were observed, compared to model, PC and NC groups after oral administration of INS/CMCD-g-CMCs. The results testified that the treatment with different doses of INS/CMCD-g-CMCs could efficiently adjust serum lipids metabolism with type 2 diabetes.

3.8. Effect of INS/CMCD-g-CMCs on antioxidant enzymes activities and inflammatory factor in liver and kidney

Many antioxidant enzymes in mammals is playing the vital role in reducing oxidative stress. As a notable biomarker for lipid peroxidation, MDA is a toxic by product of lipid oxidation. The levels of serum MDA and GPT are closely reflects the extent of hepatocellular injury. SOD and CAT, the common antioxidant enzymes in mammals, play significant roles in alleviating the oxidant stress in vivo [41,42]. As shown in Table 2, compared with the control group, significantly ($p < 0.01$) decreased of SOD, CAT and increased of MDA, GPT were observed in model, PC and NC groups, indicating severe oxidative stress occurred in liver. However, oral supplementation of INS/CMCD-g-CMCs, the activities of SOD and CAT in all the Gel groups were increased significantly ($p < 0.01$), and the content of MDA and GPT were decreased significantly ($p < 0.01$). As expected, the results suggested that INS/CMCD-g-CMCs could regulate levels of SOD, MDA, CAT, and GPT in diabetic mice in a dose-dependent manner, and oxidative stress induced by hyperglycemia was significantly reduced.

Previous study supported the role of cytokines in the inflammatory and immune responses mediating the pathogenesis of diabetes mellitus [43].As shown in Fig. 3C, compared with control group, the values of NO in the model, PC and NC groups were significantly ($p < 0.01$) higher. However, the level of NO in the Gel-treated groups decreased effectively compared with that in the model, PC and NC groups. Especially the

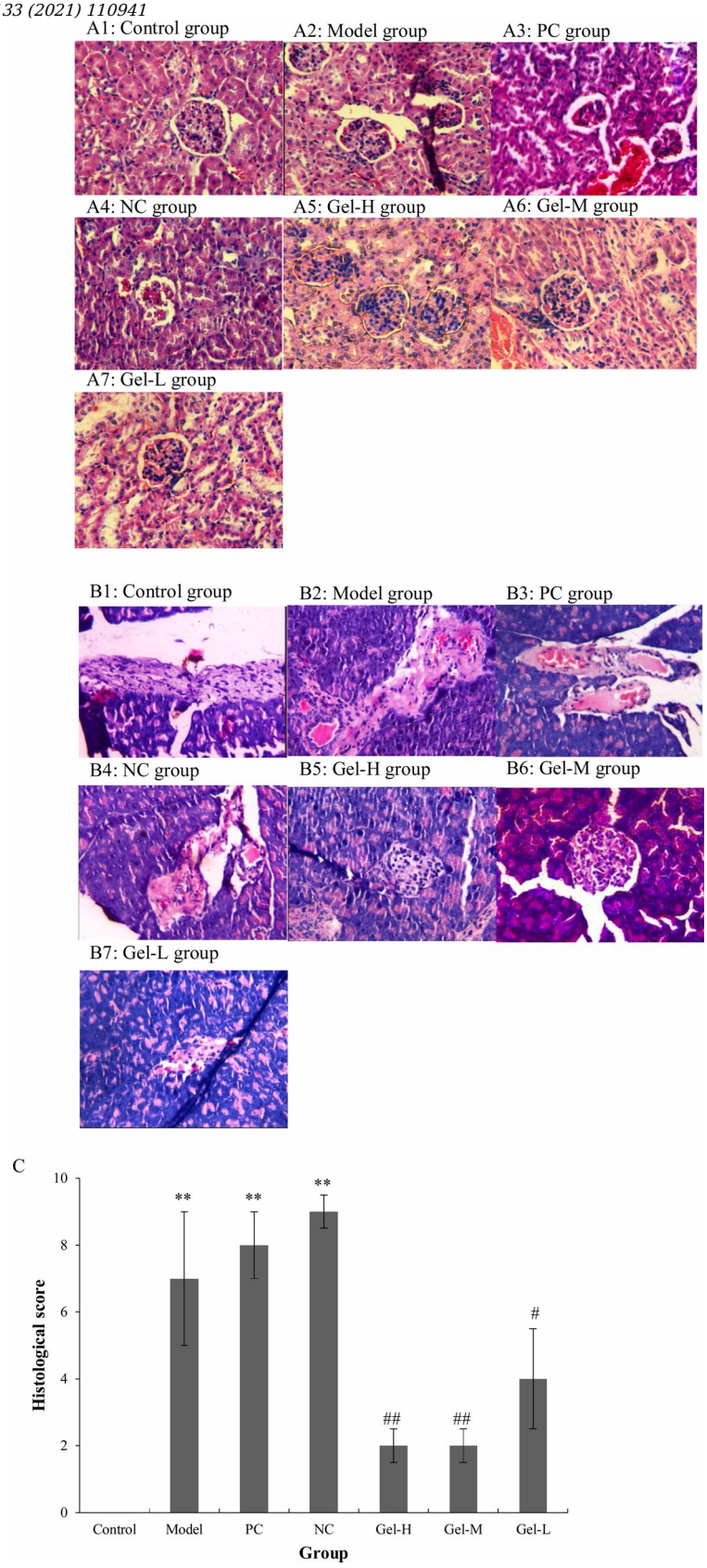

Fig. 4. Effect of INS/CMCD-g-CMCs on kidney and pancreas against damages in diabetic mice.(A1-A7) kidney (H&E stain, 400×); (B1-B7) pancreas (H&E stain, 400×); (C) histological score. Data are expressed as means ± SD (n = 10).*P < 0.05, **P < 0.01vs control; #P < 0.05, ##P < 0.01vs model.

Gel-H group became closer to the control group. Fig. 3D presented the levels of TNF-α in the kidney of mice after 4 weeks administration. The results revealed that compared with control group, the values of TNF-α in the model, PC and NC groups were significantly ($p < 0.01$) higher. The INS/CMCD-g-CMCs markedly suppressed the decreased TNF-α in the kidney of diabetes mice. Taken together, these data suggest that INS/CMCD-g-CMCs functionally improved spontaneous autoimmune diabetes and prevented the production of inflammatory factor in the type 2 diabetic mouse model.

3.9. Histological analysis of pancreas and kidney

H&E staining of the pancreas and kidney among different groups were demonstrated in Fig. 4. Diabetic nephropathy is a complication of diabetes, which is the main cause of morbidity and mortality in type 2 diabetic mice [44]. As shown in Fig. 4A1, in the normal mice, the structure of kidney was steady clear. Compared with the kidney of normal mice, the kidney architecture in STZ-induced diabetic mice showed severe pathological damages, such as mesangial dilatation and glomerular partial sclerosis(Fig.4 A2-A4). The INS/CMCD-g-CMCs treatment notably mitigated STZ-induced histology damages in different degrees (Fig.4 A5-A7), and the kidney histology of Gel-H group (Fig.4 A5) treated mice were recovered to normal as compared with mice in control group.

The islet structure of control group mice maintained the integrity, and the pancreatic β-cell was clearly visible [12] (Fig.4 B1). By contrast, the pancreatic section of model, PC and NC groups showed that high-fat diet and STZ induced pancreatic β cell reduction, malformation, depauperation, focal necrosis and hyperemia in the pancreas.(Fig.4 B2-B4).However, after the oral administration of INS/CMCD-g-CMCs, the structure of pancreatic β-cell was steady clear, with showed an higher amounts and a better islet structure, and pancreatic β-cell damage was alleviated in Gel-L, Gel-M and Gel-H group (Fig.4 B5-B7). Corroborating these results, it was proved that INS/CMCD-g-CMCs were useful for the treatment of pancreas and kidney damages. Histological score analysis also revealed that INS/CMCD-g-CMCs significantly reduced the histopathologic damage (Fig. 4C).

4. Conclusion

In summary, the effects of antidiabetic and potential hypoglycemic mechanism with long-term administration of oral INS/CMCD-g-CMCs in high-fat diet and STZ-induced type 2 diabetic mice were demonstrated in this study. The long-term treatment with different doses of INS/ CMCD-g-CMCs was effective on alleviating the symptoms of polyphagia, polydipsia, polyuria, and weight loss in diabetic mice. The INS/ CMCD-g-CMCs significantly improved obesity-induced hyperglycemia and insulin resistance. Specifically, the FBG, FINS and HOMA-IR were decreased, and OGTT and ISI were improved. Oral administration of INS/CMCD-g-CMCs showed significant anti-hyperlipidemia activity due to adjusted the levels of TC, TG, LDL-C, HDL-C and FFA, and significantly treated diabetic nephropathy by reducing the level of BUN and CRE. These results suggested that INS/CMCD-g-CMCs played an important role in improving the lipid metabolism and alleviating the symptoms of diabetic complications. In addition, a range of biochemical indicators, such as increased antioxidant enzyme activity, decreased cytokines, inflammation, and histopathological analysis confirmed that INS/CMCD-g-CMCs could protect the tissues against oxidative damages induced by hyperglycemia in the type 2 diabetic mice, possess excellent anti-inflammatory capacity, and mitigate histology damages of pancreas and kidney, which could be potential to reduce risks of diabetes complications. All the above results suggested that INS/CMCD-g-CMCs could be a promising drug candidate as effective treatment of T2DM.

Currently available therapeutic approaches for treating obesity induced T2DM have a number of limitations, such as adverse effects and high rates of secondary failure, thus oral insulin delivery exhibited great potential as an anti-hyperglycemic and anti-hyperlipidemia agent in type 2 diabetic mice. Our previous studies showed that in vitro INS/ CMCD-g-CMCs exhibited potent and obvious effect on excellent sustained-release ability of insulin and intestinal absorption after oral administration. Besides, in vivo INS/CMCD-g-CMCs exhibited effective, non-toxic and moderate hypoglycemic effect but without side effects compared with modern synthetic hypoglycemic drugs and insulin subcutaneously. Furthermore, all results of this study suggested that long- term treatment of INS/CMCD-g-CMCs played an important role in maintenance of near normal blood glucose levels and alleviating the symptoms of diabetic complications. Therefore, it is reasonable to believe that long-term administration of oral INS/CMCD-g-CMCs could be as an effective drug to treat diabetes and it may have a positive therapeutic effect on patients with T2DM and diabetic complications, which can help for achieving the therapeutic goal of T2DM in future.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people ororganizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled. We confirm that the mentioned received grants in the "Acknowledgement" section, did not lead to any conflicts of interest regarding the publication of this manuscript.

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